IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Terry et al.	Group Art Unit: 1638
Application No. 09/365,349	Examiner: ~~Ibrahim, M.~~ (Nelson, Amy)
Filed: July 30, 1999	Attorney Docket No. B99-085
For: *Heavy Metal Phytoremediation*	CERTIFICATE OF TRANSMISSION I hereby certify that this corr. is being transmitted by fax to the Comm. for Patents, Washington, D.C. 20231 at 703 746-4993 on October 17, 2003. 10 Oct 20 872-9306 Signed _____ Richard Osman

RECEIVED
CENTRAL FAX CENTER
OCT 2 1 2003
OFFICIAL

RESPONSE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Examiner,

The pending rejections and Decision dated July 31, 2003 rely on Noctor et al.'s reference to "preliminary experiments" wherein ECS-overexpressing poplars and non-transformed poplars accumulated Cd to a similar extent. The enablement rejection is expressly premised on an assumption: that "it would require an undue amount of experimentation to produce hyperaccumulating plants other than Brassica plants without further guidance from applicants as to why the construct produced a hyperaccumulating Brassica plant but failed to produce a hyperaccumulating poplar." Decision, p.14, lines 12-16.

As we explained in our Reply Brief, we do know why Noctor et al.'s preliminary experiments did not show hyper-accumulation:

> Noctor et al. did not have the benefit of our Specification, which teaches how to make the claimed hyper-accumulating plants, including hyper-accumulating poplars. Noctor et al reports that in unpublished "preliminary experiments" they failed to obtain hyper-accumulating poplars. We do not know how Noctor et al. did their experiments, so it is not possible for us to determine why they failed: we do not know in what form they provided the Cd, we do not know whether their poplars were subject to other variables that would have interfered with accumulation, we do not know how they made their transformants,

> we do not know whether their preliminary experiments were based on one or two anomalous plants, we do not know if their soil had other toxins or confounding microorganisms that may have independently depleted the supplemented Cd, etc. It is possible that the results of Noctor et al. are based on experimental error or contaminated materials. On the other hand, it is possible that they result merely from an insufficient sample size – had they generated sufficient data, they may well have obtained hyper-accumulators.
> Reply Brief p.2, lines 13-25; also quoted in Decision, para. bridging p.11 and 12.

Indeed, the same laboratory subsequently published their completed experiments (Arisi et al., Physiol Plant 109, 143-9, 2000, enclosed). When fully reported, their ECS-overexpressing poplar did indeed provide higher cadmium accumulation than corresponding untransformed plants. (Arisi et al., supra; see abstract; para. bridging col. 1 and 2 of p.145; Fig.1). Of course, this full report also had the benefit of the subject Applicant's teachings, as reported in Zhu et al. Plant Physiol 119, 73-79, 1999 and Zhu et al., Plant Physiol 121, 1169-1177, as cited, inter alia, on p.144, col.1, lines 34-37 of Arisi et al., supra.

Contrary to the premise of the pending enablement rejection, our methods produce a hyperaccumulating poplar as readily as they produce a hyperaccumulating Brassica plant. The pending rejection is contrary to evidence now of record.

We attempted to provide this reference to the Board in a Request for Rehearing; however, the Board refused to consider evidence not previously made of record (Decision dated Sept 30, 2003, p.2, lines 5-8). This submission is intended to make the reference of record.

We petition for and authorize charging our Deposit Account No.19-0750 all necessary extensions of time. The Commissioner is authorized to charge any fees or credit any overcharges relating to this communication to our Dep. Acct. No.19-0750 (order no.B99-085).

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

Richard Aron Osman, J.D., Ph.D., #36,627
Tel(650)343-4341; Fax(650) 343-4342

encl. Arisi et al., Physiol Plant 109, 143-9, 2000 (7 p.)
RCE Transmittal (1p)

PHYSIOLOGIA PLANTARUM 109: 143–149. 2000
Printed in Ireland—all rights reserved

ISSN 0031-9317

Responses to cadmium in leaves of transformed poplars overexpressing γ-glutamylcysteine synthetase

A.-C. M. Arisi[a,1], B. Mocquot[b], A. Lagriffoul[b], M. Mench[b], C. H. Foyer[c]* and L. Jouanin[a]

[a]Laboratoire de Biologie Cellulaire, INRA, Route de St Cyr, F-78026 Versailles cedex, France
[b]Unité Agronomie, INRA Bordeaux, BP 81, F-33883, Villenave-d'Ornon cedex, France
[c]Biochemistry and Physiology Department, IACR-Rothamsted, Harpenden, Hertfordshire, AL5 5JQ, UK
[1]Present address: Universidade Federal de Santa Catarina, CCA, Depto Ciência e Tecnologia de Alimentos, Cx Postal 476, 88040-900, Florianópolis-SC, Brazil
*Corresponding author: e-mail, christine.foyer@bbsrc.ac.uk

Received 15 September 1999; revised 19 January 2000

Poplars overexpressing a bacterial γ-glutamylcysteine synthetase (γ-ECS) in the cytosol (lines ggs11 and ggs28) had a 30-fold increase in foliar γ-ECS activity relative to untransformed controls. Foliar γ-glutamylcysteine (γ-EC) was increased by 10-fold while foliar glutathione accumulation increased by up to 3.5-fold in the leaves of the transformants. Untransformed and transformed poplars were grown with different soil concentrations of cadmium (0–1100 μg g^{-1} soil) for 2 weeks. Cadmium accumulated in the leaves of both transformed and untransformed poplars and growth was inhibited. Growth inhibition and foliar cadmium accumulation were greatest at the highest soil cadmium concentrations in all lines. Exposure to cadmium enhanced the foliar cysteine, γ-EC and glutathione pools in all lines but less glutathione was present in the leaves of the untransformed controls than the transformants under all growth conditions. Cadmium-induced changes in the activities of malic enzyme, isocitrate dehydrogenase and guaiacol peroxidase were less pronounced in the leaves of the transformed poplars overexpressing γ-ECS than in the untransformed controls. Glutamate dehydrogenase and glutathione reductase activities were unchanged by exposure to cadmium. We conclude that overexpression of γ-ECS activity and foliar glutathione accumulation in transformed poplar allows greater tissue cadmium accumulation but has only a marginal effect on cadmium tolerance in poplar.

Introduction

Cadmium is a pollutant that accumulates in soil as a result of industrial processes or intensive use of fertilisers in agriculture. Its phytotoxicity is related to its reactivity with O-, N- and S-containing ligands (Van Assche and Clijsters 1990a). Cd inhibits photosynthesis (Clijsters and Van Assche 1985) but stimulates respiration. The activities of the tricarboxylic acid cycle and of other pathways of carbohydrate utilisation are induced by Cd accumulation in leaves. This is related to increased demand for ATP production by oxidative phosphorylation to compensate for deficits in photophosphorylation (Ernst 1980). In particular, the activities of isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), malic enzyme (ME), glucose 6-phosphate dehydrogenase and peroxidases (POD) are increased following Cd exposure (Van Assche and Clijsters 1990a).

Cd induces the synthesis of cysteine-rich peptides with the general structure $(\gamma\text{-EC})_nG$, called phytochelatins (Rauser 1995), and of other thiol peptides $(\gamma\text{-EC})_n$ and $(\gamma\text{-EC})_nE$ (Meuwly et al. 1995). Phytochelatins form complexes with Cd in the cytosol and are important in subsequent Cd sequestration in the vacuoles (Ortiz et al. 1995). They participate in the maintenance of cellular metal homeostasis (Zenk 1996) and are involved in limiting the transport of heavy metal ions from roots to shoots (Galli et al. 1996). Phytochelatins are synthesised by γ-EC dipeptidyl transpeptidase from the precursor reduced glutathione (GSH; Grill et al. 1989). GSH is synthesised by two sequential reactions,

Abbreviations – γ-ECS, γ-glutamyl cysteine synthetase; GDH, glutamate dehydrogenase; GR, glutathione reductase; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, oxidised glutathione; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; POD, peroxidase.

catalysed by γ-glutamyl cysteine synthetase (γ-ECS) and glutathione synthetase (GS) in the chloroplasts and cytosol of plant cells (Noctor et al. 1998a,b).

In addition to phytochelatins, metallothioneins are cysteine-rich peptides that play a major role in metal detoxification (Zhou and Goldsbrough 1994). Overexpression of metallothionein genes in transformed plants has been used to study Cd tolerance. Expression of a mammalian metallothionein cDNA in transformed tobacco plants resulted in improved Cd resistance (Pan et al. 1994) and in reduced Cd accumulation in leaves (Maiti et al. 1991, Elmayan and Tepfer 1994). Several studies have shown that tolerance to Cd is also related to GSH accumulation in leaves and an increased capacity of GSH synthesis. A Cd-sensitive mutant of *Arabidopsis thaliana*, deficient in the ability to sequester Cd, was shown to have very low glutathione contents (Howden et al. 1995). GSH was decreased in the roots of pea plants cultivated with Cd (Klapheck et al. 1995). In maize, Cd exposure led to decreased GSH and increased γ-EC in both roots and shoots (Rauser et al. 1991, Rüegsegger and Brunold 1992) and to increases in the maximal extractable γ-ECS activity of roots (Rüegsegger and Brunold 1992). GSH depletion and γ-EC accumulation were also observed in parsley and tobacco cell cultures following Cd treatment. γ-ECS and GS activities were found to increase in tobacco cell cultures treated with Cd (Schneider and Bergmann 1995). Cultured tomato cells, selected for increased Cd tolerance, had increased γ-ECS activity (Chen and Goldsbrough 1994). Cd exposure of *Arabidopsis* plants activated transcription of the genes for glutathione synthesis (Xiang and Oliver 1998). Furthermore, buthionine sulphoximine, an inhibitor of γ-ECS, inhibited phytochelatin accumulation (Grill et al. 1987) and enhanced the Cd-dependent growth inhibition in plants (Gussarsson et al. 1996). Transformed Indian mustard plants overexpressing either GS (Zhu et al. 1999a) or γ-ECS (Zhu et al. 1999b) accumulated more Cd than untransformed plants and showed enhanced tolerance.

We have produced transformed poplars overexpressing the *Escherichia coli* γ-ECS in the cytosol (Noctor et al. 1996, Arisi et al. 1997, Noctor et al. 1998a). These transformed plants have constitutively increased foliar glutathione contents compared with untransformed poplars, whereas GS overexpression did not modify foliar GSH contents of poplars (Strohm et al. 1995, Noctor et al. 1998b). γ-ECS is the rate-limiting enzyme for glutathione biosynthesis in poplars. To investigate the role of enhanced γ-ECS activity and glutathione in protection against Cd-induced inhibition of metabolism, transformed and untransformed poplars were grown for 2 weeks at different Cd concentrations. This is the first report of Cd-induced changes in foliar thiol contents, the activities of POD and of enzymes related to carbon utilisation in transformed plants with increased γ-ECS activity.

Materials and methods

Plant material

Untransformed (WT) and transformed hybrid poplars (*Populus tremula* × *P. alba*; Institut National de la Recherche Agronomique No. 717-1-B4, Versailles, France), overexpressing γ-glutamylcysteine synthetase in the cytosol, were micropropagated in vitro. The two lines (ggs11 and ggs28) of transformed poplar used in the present work had high extractable foliar γ-ECS activities relative to untransformed poplar (Arisi et al. 1997). The plants were transferred to pots containing artificial soil (70% quartz sand, 20% kaolin, 10% ground peat, 0.5% $CaCO_3$) and introduced in the greenhouse, where they were regularly watered with nutrient solution. After 6 weeks in the greenhouse, plants were put into bigger pots containing 475 g of artificial soil per pot and then transferred to a controlled environment chamber with a 16-h photoperiod (300 μmol m^{-2} s^{-1} irradiance), 25/20°C day/night and 75% relative humidity. Plants were fed with nutrient solution (1 m*M* $NH_4H_2PO_4$, 5.6 m*M* KNO_3, 4.0 m*M* $Ca(NO_3)_2$, 2.0 m*M* $MgSO_4$, 46.3 μ*M* H_3BO_3, 9.1 μ*M* $MnCl_2$, 64.7 μ*M* $FeSO_4$, 0.3 μ*M* $CuSO_4$, 0.76 μ*M* $ZnSO_4$, 0.52 μ*M* H_2MoO_4) every 3 days and watered several times a day to maintain the water status at between 50 and 70% of the retention capacity. For analysis of foliar metabolism in all the following experiments, only the youngest mature leaves were used.

Cadmium treatment

One week after the transfer to the controlled environmental chamber, the height of the plants was measured and the 5th leaf from the top was tagged. Then, $Cd(NO_3)_2$ solution was added to the soil in order to obtain the following Cd concentrations in the soil: 0, 100, 300, 500, 700, 900, 1100 μg g^{-1} dry weight. After 2 weeks of Cd treatment, the height of the plants was measured and the number of new leaves that appeared at the top was determined. Three plants at each Cd concentration from each poplar line (WT, ggs11 and ggs28) were harvested and washed in distilled water. Each plant was divided into roots, bottom leaves (below and including the tagged 5th leaf), medium leaves (4th, 3rd and 2nd, which had enlarged during the treatment), upper leaves (3 following leaves which appeared and enlarged during the treatment), top leaves (small leaves that had appeared above the upper leaves) and remaining shoot and petioles. For each treatment, roots, medium leaves and upper leaves were pooled and then cut with ceramic scissors. The resulting small pieces were sampled (0.5 g fresh weight) and the samples were immediately frozen in liquid N_2 and stored at −80°C for thiol and enzyme measurements. The remaining leaf pieces and other lower parts were weighed and oven-dried at 80°C to constant weight for determining dry weight and cadmium content.

Metal analysis

Oven-dried plant samples were heated for several hours at 470°C in an oven. The resulting ashes were solubilised in 5% HNO_3 (50 ml final volume per 0.5 g dry weight). Cd concentration in digested solutions was determined by inductively coupled plasma emission (Varian Liberty 200, Victoria, Australia). Quality control for plant analysis was performed by analysing blanks and certified reference material (Ryegrass BCR 281, Community Bureau of Reference, Commission of the European Communities) using the same method in triplicate.

Determination of thiols

Cysteine, γ-glutamylcysteine (γ-EC) and GSH were extracted in acid from samples frozen at −80°C. Samples of root and shoot tissues (0.5 g fresh weight) were ground in a mortar with liquid nitrogen, then, 100 mg insoluble polyvinylpyrrolidone and 5 ml 0.1 *M* HCl, 1 m*M* EDTA were added. Thiol contents were determined fluorimetrically as monobromobimane derivatives following separation by reverse-phase HPLC, as described in Arisi et al. (1997). Oxidised glutathione (GSSG) was assayed as described in Foyer et al. (1995), after the method of Griffith (1980).

Enzyme assays

Frozen material (0.5 g fresh weight) was homogenised with an ice cold mortar and pestle in 2.5 ml of 0.1 *M* Tris-HCl buffer (pH 7.8), containing 1 m*M* dithiothreitol and 1 m*M* EDTA. The homogenate was squeezed through a nylon mesh and centrifuged at 12000 *g* at 4°C for 10 min. The supernatant was collected and the activities of ME, ICDH and GDH were measured spectrophotometrically with a Varian Cary 1E spectrophotometer as described by Van Assche et al. (1988). POD was assayed by the method of Van Assche and Clijsters (1990b) and glutathione reductase (GR) was assayed as described by Foyer and Halliwell (1976).

Anionic (iso)peroxidases were separated by polyacrylamide gel electrophoresis on 7.5–20% gradient gels. POD activity was detected by incubation with 0.04% benzidine and 0.006% (v/v) H_2O_2 for 1.5 h at 37°C (Van Assche and Clijsters 1990b) and bands of POD activity quantitated by densitometry at 632 nm.

Determination of relative growth and statistical analysis

The height of each shoot was measured from the plant/soil interface to the uppermost leaf. Relative growth (RG) was determined as the final plant height minus the initial height divided by the initial height and expressed as a percentage. All lines were fitted using software from Biosoft, Cambridge, UK applying the least-squares method.

Results

Leaf Cd concentration and plant growth

Poplars overexpressing γ-ECS in the cytosol (ggs11 and ggs28) and untransformed (WT) poplars were grown for 2 weeks in the presence of Cd (0–1100 μg g^{-1} dry weight soil). In all plant types, foliar Cd accumulation increased in relation to the Cd content of the soil as shown in Fig. 1. Foliar Cd contents increased sharply relative to soil Cd content at low soil Cd concentrations (0–200 μg g^{-1} dry weight). For soil Cd contents between 200 and 900 μg g^{-1} dry weight, foliar Cd contents were only slightly increased. A large increase in foliar Cd occurred in plants grown at soil Cd concentrations between 900 and 1100 μg g^{-1} dry weight. At high soil Cd concentrations (1100 μg g^{-1} dry weight), the transformed poplars tended to show higher foliar cadmium accumulation than the untransformed controls. Similar results were obtained for upper and lower leaves on the trees.

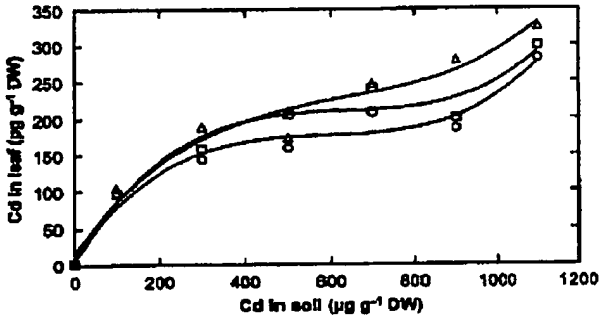

Fig. 1. Foliar Cd accumulation in untransformed (○) and transformed (△, ggs11; □, ggs28) poplars after 2 weeks growth in the presence of different soil Cd concentrations.

Cd accumulation caused decreased growth in all plant types (Fig. 2A). Cd-dependent growth inhibition was greatest in plants grown with soil Cd concentrations above 500 μg g^{-1} dry weight (corresponding to 150–200 μg g^{-1} dry weight in the leaves) and was most marked in transformed poplars. The effect of Cd on relative growth rate was similar in all leaves (Fig. 2B) indicating that Cd-induced effects on total new leaf biomass were comparable in all plants.

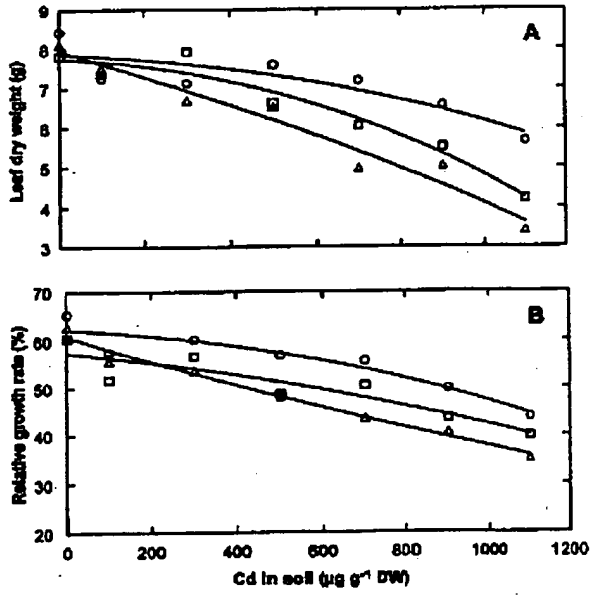

Fig. 2. Foliar dry weight (A) and shoot relative growth rates (B) of untransformed (○) and transformed (△, ggs11; □, ggs28) poplars after 2 weeks growth in the presence of different soil Cd concentrations.

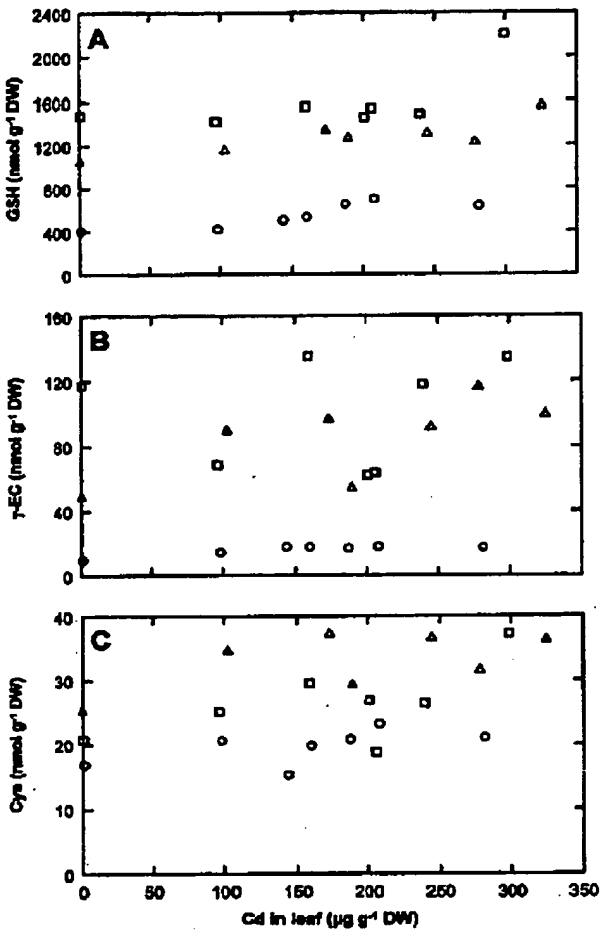

Fig. 3. Foliar thiol accumulation in untransformed (O) and transformed (Δ, ggs11; □, ggs28) poplars relative to foliar leaf Cd contents. Plants were harvested after 2 weeks growth in the absence and presence of different Cd concentrations (A) foliar glutathione (GSH) (B) γ-glutamyl cysteine (γ-EC) and (C) cysteine (Cys). The results presented are the mean values of at least two determinations performed on pooled leaf samples from 3 individual plants.

Effect of Cd on thiol contents

Leaves from transformed poplars overexpressing γ-ECS (lines ggs11 and ggs28) had 2.5- and 3.5-fold more GSH in their leaves, respectively, than those of untransformed (WT) poplars in the absence of cadmium (Fig. 3; Arisi et al. 1997). The roots of these transformed plants also had approximately 3 times more GSH (A. C. M. Arisi 1997. Thesis, Université de Paris-Sud UFR Scientifique d'Orsay, France). After 2 weeks growth with Cd, the GSH content of the roots was similar in all plants (data not shown) and not changed by the presence of Cd in the soil. The GSH contents of the leaves were increased, however, in all plant types. The increase was proportional to the Cd content of the leaves when the foliar Cd content was over 100 µg Cd g^{-1} dry weight leaf. At higher levels of foliar Cd accumulation, foliar GSH content in untransformed poplars increased 400–700 nmol g^{-1} dry weight of untransformed plants, but this value was much less than the GSH content of the leaves of the transformants. GSH exceeded 1000 nmol g^{-1} dry weight in lines ggs11 and ggs28 (Fig. 3). Even in the transformed poplars, GSH increased as a result of Cd exposure, in ggs11 GSH increased from 1040 to 1540 nmol g^{-1} dry weight in the presence of Cd. Foliar GSH increased only at the highest foliar Cd concentrations (300 µg g^{-1} dry weight) in line ggs28 to a maximum of 2160 nmol GSH g^{-1} dry weight. It is important to note that ggs28 plants showed the highest foliar GSH contents prior to Cd treatment and was only exceeded by ggs11 when foliar Cd reached 320 µg g^{-1} dry weight. The GSH/GSSG ratio was 9/1 in leaves for all plants under all growth conditions (Arisi et al. 1997) and was not changed by Cd treatment (data not shown).

Foliar γ-EC was up to 10-fold higher in lines ggs11 and ggs28 relative to untransformed controls (Fig. 3). Cd exposure caused a 2-fold increase of γ-EC in the untransformed poplars. However, these values were still much less than those obtained with lines ggs11 and ggs28 without the Cd treatment (50 and 120 nmol g^{-1} dry weight, respectively; Fig. 3). Foliar γ-EC contents were enhanced following Cd treatments. Similar results were obtained for upper and lower leaves (data not shown).

Transformed poplar leaves overexpressing γ-ECS generally contained more cysteine than those from untransformed plants (Arisi et al. 1997, Noctor et al. 1998a). The cysteine pool in leaves prior to Cd exposure amounted to 17, 26 and 20 nmol g^{-1} dry weight in untransformed poplars, lines ggs11 and ggs28, respectively (Fig. 3). Foliar cysteine increased with increasing foliar Cd contents in all plants (Fig. 3), especially above 150 µg Cd g^{-1} dry weight.

Effect of Cd on enzyme activities

In the upper leaves, an increase in the activities of the NAD(P)H-producing enzymes ME and ICDH was observed as Cd accumulated in the leaves (Fig. 4). The activities of these enzymes in the absence of Cd were similar in untransformed and transformed lines (Fig. 4). ME activity was increased to values 2.5-fold higher in ggs11 and ggs28 and 4-fold higher in the untransformed poplars than leaves of plants grown in the absence of Cd. Cd-induced increases in ICDH activity were 2- and 3-fold, respectively, in transformed lines and in untransformed controls.

Foliar POD activity (Fig. 4) was lower in the transformed lines (105 mU mg^{-1} protein) than the untransformed plants (140 mU mg^{-1} protein) in the absence of Cd. An increase in POD activity was observed in untransformed poplars and ggs28 at foliar Cd concentrations above 150 µg g^{-1} dry weight, and in ggs11 at foliar Cd concentration of 100 µg g^{-1} dry weight. Cd-induced changes in total foliar POD activity were not reflected by a change in the pattern of isoenzyme activity bands revealed by activity staining on gels following electrophoresis, even at the highest Cd con-

centrations (data not shown). The intensity of all POD activity bands was increased uniformly (data not shown).

The activities of GDH and GR were not modified by Cd exposure (data not shown).

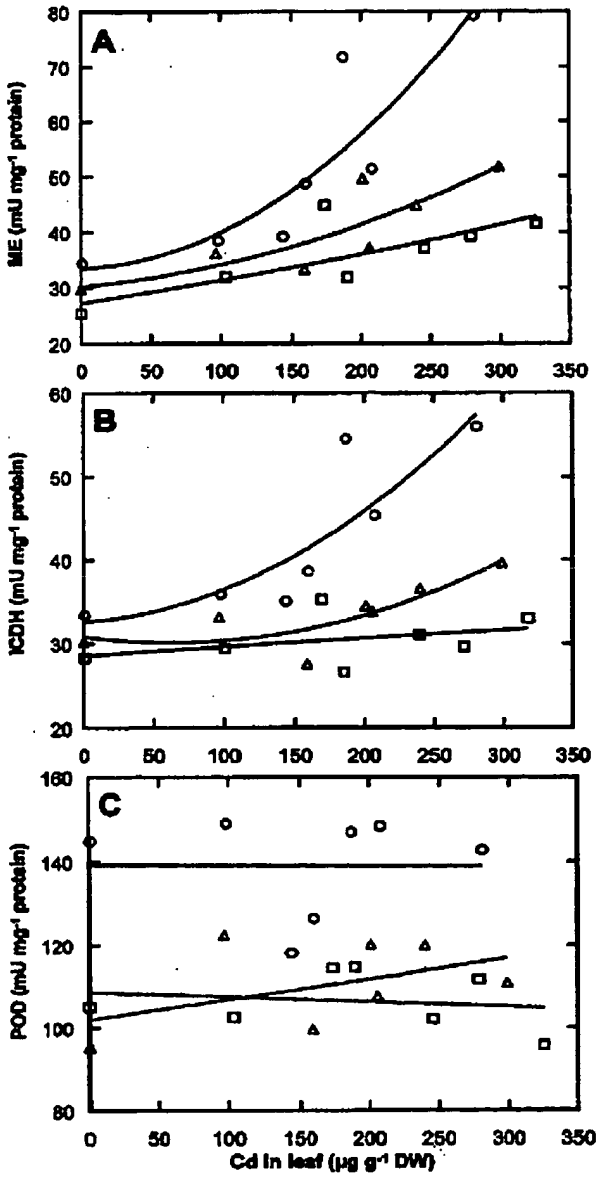

Fig. 4. Foliar activities of (A) malic enzyme (ME) (B) isocitrate dehydrogenase (ICDH) and (C) gualacol peroxidase (POD) from untransformed (○) and transformed (△, ggs11; □, ggs28) poplars relative to foliar leaf Cd contents. Plants were harvested after 2 weeks growth in the presence of increasing Cd concentrations. The results presented are the mean values of at least two determinations performed on pooled leaf samples from 3 individual plants.

Discussi n

The effects of cadmium on growth and metabolism were studied in untransformed poplars and in transformed poplars overexpressing γ-ECS with constitutively enhanced tissue GSH contents (Noctor et al. 1998a). Leaves of the transformed lines had a slightly higher Cd content than those of untransformed controls, particulary at high soil Cd concentrations (Fig. 1). The accumulation of Cd was associated with a proportionally greater Cd-induced inhibition of growth in the transformants (Fig. 2). Analysis of morphological markers, such as biomass and relative growth rate, suggests that the untransformed controls tolerated more Cd than the transformed lines. Transformed Indian mustard overexpressing either GS or γ-ECS accumulated more Cd than untransformed plants and were more tolerant to Cd (Zhu et al. 1999a,b). While the ME, POD and ICDH data obtained in the present work suggest that metabolism in the γ-ECS transformants is less perturbed by Cd than in the untransformed controls, these results do not provide conclusive proof that Cd tolerance is increased by γ-ECS overexpression.

Exposure to Cd led to increases in foliar γ-EC and GSH accumulation in all poplar lines, irrespective of the GSH content of the leaves in the absence of Cd. An increase in foliar GSH was observed in maize leaves at leaf Cd concentrations above 3.4 µg g^{-1} dry weight (Lagriffoul et al. 1997); but, in poplars, the increase in GSH occurred only at much higher Cd values. The factors responsible for the Cd-induced increases in thiols merit attention, since it is clear that GSH synthesis is under multifactorial control (Noctor et al. 1998a). Treatment of maize roots and leaves with Cd led to increased γ-EC contents and enhanced rates of GSH synthesis (Rüegsegger and Brunold 1992). Similar observations were reported on exposure of cultured cells to Cd (Schneider and Bergmann 1995). In these previous studies, the increases in thiols were accompanied by increases in the extractable activities of γ-ECS (Rüegsegger and Brunold 1992) and GS (Schneider and Bergmann 1995). Increases in γ-ECS are likely to be particularly important, since overexpression studies strongly suggest that the major control over GSH accumulation resides with this enzyme (Noctor et al. 1996, Arisi et al. 1997, Noctor et al. 1998a,b). In both animal and plant cells, Cd treatment induced increases in γ-ECS gene transcription (Hatcher et al. 1995, Xiang and Oliver 1998). Thus, up-regulation of γ-ECS may well have contributed to the observed increases in GSH in untransformed poplars. However, the transformants used in the present work have extractable γ-ECS activities which, even in the absence of Cd, are higher than those induced by Cd in maize plants and tobacco cells (Rüegsegger and Brunold 1992, Schneider and Bergmann 1995). It is unclear, therefore, whether the slight increases in thiols in the ggs transformants can be explained by up-regulation of γ-ECS. Overexpression of γ-ECS causes the control of GSH synthesis to shift to the second enzyme, GS (Noctor et al. 1998a). It is possible that induction of this enzyme activity, as observed in Cd-exposed pea roots (Rüegsegger et al. 1990) and tobacco cells (Schneider and Bergmann 1995), may have contributed to the Cd-induced GSH accumulation in the

poplar transformants. In support f this view, Zhu et al. (1999a) have demonstrated that overexpression of GS enhances cadmium accumulation in *Brassica juncea*. Thus, γ-ECS would appear to be the rate-limiting step in glutathione biosynthesis in the absence of Cd, while exposure to Cd shifts the equilibrium so that GS also limits GSH production.

Whatever the factors responsible for the increases in thiols, the present data confirm that induction of GSH biosynthesis seems to be an early inducible mechanism of protection against toxic heavy metal accumulation. This protection would presumably be manifested through an effect of increased foliar GSH on the synthesis of phytochelatins. Phytochelatins are known to inactivate Cd in the cytoplasm and facilitate the transport of Cd into the vacuole. Although direct measurements of foliar phytochelatin contents were beyond the scope of the present work, the high GSH content of leaves and roots in the transformed plants should confer a greater capacity for phytochelatin synthesis, enabling formation of more thiolate bonds with Cd. In *Brassica juncea* exposed to Cd, the expression of γ-ECS was closely correlated with phytochelatin synthesis (Haag-Kerwer et al. 1999). The increased accumulation of Cd in the leaves of the transformants suggests that there is some enhancement of phytochelatin synthesis in the leaves of these plants relative to controls.

The activities of enzymes associated with energy metabolism and regeneration of reducing power (NADPH and NADH) were increased by Cd treatment. ME and ICDH activities were similar in untransformed and transformed plants in the absence of Cd, suggesting that the transformation procedures have not modified the general energy metabolism of the poplar cells. The Cd-induced increases in ME and ICDH activities were higher in the leaves of the untransformed poplars at the highest foliar Cd concentrations. Increases in enzyme activity were observed at foliar Cd concentrations above 150 μg g^{-1} dry weight in all plant types. This value is high compared with values of Cd that induce increases in enzyme activity in bean leaves (4.6 and 5.5 μg g^{-1} dry weight, respectively, for ME and ICDH; Van Assche et al. 1988). Conversely, maize leaves showed decreased ICDH activity at Cd concentrations above 22 μg g^{-1} dry weight and no significant Cd-induced changes in ME activity were observed (Lagriffoul et al. 1997).

An increase in POD activity above foliar Cd concentrations of 150 μg g^{-1} dry weight was observed. As for the activities of other enzymes, the increase in POD activity was highest in the untransformed poplars. POD activity was found to be induced in bean and maize leaves at lower Cd concentrations than in poplar: 5.5 μg g^{-1} dry weight in bean (Van Assche et al. 1988) and 3–5 μg g^{-1} dry weight in maize (Lagriffoul et al. 1998). In poplars, as in maize, no induction of new POD isoenzymes was found. In contrast, two minor novel bands of POD activity were observed in bean leaves following Cd treatment (Van Assche and Clijsters 1990a). It is interesting to note that no changes in GR activity were observed in Cd-treated plants, in contrast to previous observations in maize (Lagriffoul et al. 1997). GR activity in poplars may be sufficiently high to maintain the balance between xidised and GSH in the presence of Cd, requiring no further induction of GR activity. Similarly, no Cd-induced changes in GDH activity were observed. This latter observation is similar to previous studies in maize (Lagriffoul et al. 1997), but in contrast to work in bean leaves, where an induction of GDH activity was observed following Cd treatment (Van Assche et al. 1988). Overexpression of either γ-ECS or GS increased Cd accumulation and tolerance in Indian Mustard. In transformed poplars, enhanced tissue γ-EC and GSH do not confer increased tolerance to Cd in terms of amelioration of growth. However, overexpression γ-ECS markedly decreased Cd-dependent activation of enzymes such as ME, POD and ICDH in the transformed poplars. Metabolism was, therefore, less perturbed by Cd in leaves overexpressing γ-ECS than in untransformed controls, probably because Cd was complexed in a non-toxic form in the transformants.

The leaves of the transformed poplars contained somewhat more Cd than the untransformed controls, suggesting potential importance for phytoremediation. The results presented here largely agree with those of Zhu et al. (1999a,b). We conclude that simultaneous overexpression of γ-ECS and GS, to enhance GSH production and accumulation even further (Noctor and Foyer 1998), is a promising strategy for improved heavy metal phytoremediation capacity.

Acknowledgements – A. C. M. Arisi was supported by a doctoral fellowship from CAPES, Ministry of Education, Brazil. The authors gratefully acknowledge the expert advice and assistance of G. Noctor in the HPLC analysis of thiol compounds and for critical reading of the manuscript and LERMAUE (Bordeaux) for Cd analysis.

References

Arisi ACM, Noctor G, Foyer CH, Jouanin L (1997) Modification of thiol contents in poplar (*Populus tremula* × *P. alba*) overexpressing enzymes involved in glutathione synthesis. Planta 203: 362–372

Chen J, Goldsbrough PB (1994) Increased activity of γ-glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. Plant Physiol 106: 233–239

Clijsters H, Van Assche F (1985) Inhibition of photosynthesis by heavy metals. Photosynth Res 7: 31–40

Elmayan T, Tepfer M (1994) Synthesis of a bifunctional metallothionein/β-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. Plant J 6: 433–440

Ernst WHO (1980) Biochemical aspects of cadmium in plants. In: Nriagu JO (ed) Cadmium in the Environment. J Wiley & Sons, New York, NY, pp 639–653

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. Planta 133: 21–25

Foyer CH, Souriau N, Perret S, Lelandais M, Kunert KJ, Pruvost C, Jouanin L (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. Plant Physiol 109: 1047–1057

Galli U, Schüepp H, Brunold C (1996) Thiols in cadmium- and coper-treated maize (*Zea mays* L.). Planta 198: 139–143

Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106: 207–212

Grill E, Winnacker EL, Zenk MH (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA 84: 439–443

Grill E, Löffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesised from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad Sci USA 86: 6838–6842

Gussarsson M, Asp H, Adalsteinsson S, Jensén P (1996) Enhancement of cadmium effects on growth and nutrient composition of birch (*Betula pendula*) by buthionine sulphoximine (BSO). J Exp Bot 47: 211–215

Haag-Kerwer A, Schafer HJ, Heiss S, Walter C, Rausch T (1999) Cadmium exposure in *Brassica juncea* causes a decline in transpiration rate and leaf expansion without effect on photosynthesis. J Exp Bot 50: 1827–1836

Hatcher EL, Chen Y, Kang YJ (1995) Cadmium resistance in A542 cells correlates with elevated glutathione content but not antioxidant enzymatic activities. Free Radic Biol Med 19: 805–812

Howden R, Andersen CR, Goldsbrough PB, Cobbett CS (1995) A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol 107: 1067–1073

Klapheck S, Schlunz S, Bergmann L (1995) Synthesis of phytochelatins and homo-phytochelatins in *Pisum sativum* L. Plant Physiol 107: 515–521

Lagriffoul A, Mocquot B, Mench M (1997) Assessment of the phytoxicity of cadmium-contaminated soils by an ecotoxicological biotest using maize metabolic biomarkers. In: Iskandar IK, Hardy SE, Chang AC, Pierzynski GM (eds) Proceedings of the Fourth International Conference on the Biogeochemistry of Trace Elements. Berkeley, CA, pp 557–558

Lagriffoul A, Mocquot B, Mench M, Vangronsveld J (1998) Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress-related enzymes in young maize plants (*Zea mays* L.). Plant Soil 200: 241–250

Maiti IB, Wagner GJ, Hunt AG (1991) Light inducible and tissue specific expression of a chimeric mouse metallothionein cDNA gene in tobacco. Plant Sci 76: 99–107

Meuwly P, Thibault P, Schwan AL, Rauser WE (1995) Three families of thiol peptides are induced by cadmium in maize. Plant J 7: 391–400

Noctor G, Foyer CH (1998) Ascorbate and glutathione: Keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249–279

Noctor G, Strohm M, Jouanin L, Kunert KJ, Foyer CH, Rennenberg H (1996) Synthesis of glutathione in leaves of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing γ-glutamylcysteine synthetase. Plant Physiol 112: 1071–1078

Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998a) Glutathione: Biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49: 623–647

Noctor G, Arisi ACM, Jouanin L, Foyer CH (1998b) Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol 118: 362–372

Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270: 4721–4728

Pan A, Yang M, Tie F, Li L, Chen Z, Ru B (1994) Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants. Plant Mol Biol 24: 341–351

Rauser WE (1995) Phytochelatins and related peptides. Structure, biosynthesis, and function. Plant Physiol 109: 1141–1149

Rauser WE, Schupp R, Rennenberg H (1991) Cysteine, γ-glutamylcysteine, and glutathione levels in maize seedlings. Distribution and translocation in normal and cadmium-exposed plants. Plant Physiol 97: 128–138

Rüegsegger A, Brunold C (1992) Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. Plant Physiol 99: 428–433

Rüegsegger A, Schmultz D, Brunold C (1990) Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. Plant Physiol 93: 1579–1584

Schneider S, Bergmann L (1995) Regulation of glutathione synthesis in suspension cultures of parsley and tobacco. Bot Acta 108: 34–40

Strohm M, Jouanin L, Kunert KJ, Pruvost C, Polle A, Foyer CH, Rennenberg H (1995) Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing glutathione synthetase. Plant J 7: 141–145

Van Assche F, Clijsters H (1990a) Effects of metals on enzyme activity in plants. Plant Cell Environ 13: 195–206

Van Assche F, Clijsters H (1990b) A biological test system for the evaluation of the phytotoxicity of metal contaminated soils. Environ Pollut 66: 157–172

Van Assche F, Cardinaels C, Clijsters H (1988) Induction of enzyme capacity in plants as a result of heavy metal toxicity; dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium. Environ Pollut 52: 103–115

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10: 1539–1550

Zenk MH (1996) Heavy metal detoxification in higher plants – a review. Gene 179: 21–30

Zhou J, Goldsbrough PB (1994) Functional homologs of fungal metallothionein genes from *Arabidopsis*. Plant Cell 6: 875–884

Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N (1999a) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiol 119: 73–79

Zhu YL, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N (1999b) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol 121: 1169–1177

Edited by J. I. Sprent